PHARMACEUTICAL USES OF NAB1 AND NAB2

This invention relates to the use of gene therapy techniques in wound healing. More particularly, it relates to a new use of polynucleotides encoding NAB1 or NAB2 transcriptional repressor proteins in the down-regulation of cell proliferative disorders particularly slowing down the healing of skin to prevent hypertrophic and keloid scar formation, psoriasis, inhibition of restenosis following percutaneous trans-luminal coronary angioplasty, modulation of vessel wall calcification and inhibition of cell proliferation in cancer.

The healing of skin involves a wide range of cellular, molecular, physiological and biochemical events. During the healing process, cells migrate to wound sites where they proliferate and synthesise extracellular matrix components in order to reconstitute a tissue closely similar to the uninjured original. This activity is regulated by mediators secreted from the wound border cells such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor beta (TGF beta) and other cytokines. Beneficial effects of these agents on cells has been demonstrated both *in vitro* and *in vivo* (reviewed by Moulin, Eur. J. Cell Biol. 68; 1-7, 1995), including benefit of administering PDGF in rat models of diabetes (Brown et al J.Surg. Res. 56; 562-570, 1994).

Over the last five years numerous growth factors have been shown to accelerate cell proliferation *in vitro* and to promote wound healing in animal models. TGF beta has received the greatest attention in the context of wound repair as it promotes cell proliferation, differentiation and matrix production. TGF beta administered either topically or systemically accelerates the rate of cutaneous wound repair in animal models. (Ashcroft et al Nature Medicine, 3; 1209-1215, 1997; Sporn and Roberts J. Cell Biol. 119;1017-1021, 1997; Beck et al J. Clin. Invest. 92; 2841-2849, 1993). Likewise PDGF has been reported to promote re-epithelialisation and revascularisation in ischemic tissue and diabetic animals (Uhl et al Langenbecks Archiv fur Chirurgie-Supplement-

Kongressband 114; 705-708, 1997 and reviewed in Dirks and Bloemers Mol. Biol. Reports 22; 1-24, 1996).

The transcription factor Egr-1 (early growth response gene) is a potential regulator of over 30 genes and plays a role in cell proliferation, development and differentiation (reviewed in Liu et al Crit. Rev. Oncogenesis 7; 101-125, 1996; Khachigian and Collins Circ. Res. 81; 457-461, 1997). Egr-1 is induced upon injury to the vascular endothelium (e.g. Khachigian et al Science; 271, 1427-1431, 1996) and targets for transcriptional activation are numerous genes including EGF, platelet derived growth factor-A (PDGF-A), basic fibroblast growth factor (bFGF), induction of PDGF A, platelet derived growth factor-B (PDGF B), TGF beta, bFGF, uro-plasminogen activator (u-PA), tissue factor and insulin-like growth factor-2 (IGF-2). Blockade of Egr-1 inducible TGF beta may have application in preventing scarring. Antibodies raised against TGF beta reduce scarring in incisional wounds in rodents (Shah et al J. Cell Science; 107; 1137-1157; Shah et al Lancet; 339, 213-214, 1992).

The transcription complex that mediates vascular endothelial growth factor (VEGF) induction is dependent upon AP2 and not Egr-1 directly (Gille et al EMBO J 16; 750-759, 1997). However PDGF B directly upregulates VEGF expression (Finkenzeller Oncogene 15; 669-676, 1997). Transcription of VEGF mRNA is enhanced by a number of factors including PDGF B, bFGF, keratinocyte growth factor (KGF), EGF, tumour necrosis factor (TNF) alpha and TGF beta1. In animal models, VEGF-driven passivation of metallic stents has been shown to inhibit neo-intima formation, accelerate re-endothelialisation and increase vasomotor activity (Asahara et al Circulation; 94, 3291-3302).

VEGF expression has been reported in healing wounds and psoriatic skin, both conditions in which TGF alpha and its ligand the EGFr are upregulated. Expression of EGF induces Egr-1 (Iwami et al Am. J. Physiol. 270; H2100-2107, 1996; Fang et al Calcified Tissue International 57; 450-455, 1995; J. Neuroscience Res. 36; 58-65, 1993). There is at present anecdotal evidence that Egr-1 may activate the expression of intercellular adhesion molecule-1 (ICAM-1) in phorbol ester stimulated B lymphocytes (Maltzman et al Mol. Cell.

Biol; 16; 2283-2294, 1996) and may activate the expression of TNF alpha by virtue of the presence of an Egr-1 binding site in the TNF alpha promoter (Kramer et al Biochim. Biophys. Acta 1219; 413-421, 1994). Finally, Egr-1 knock out mice are infertile and luteinizing hormone (LH) deficient (Lee et al 273; 1219-1221, 1996) implying that the LH promoter may also be a target for Egr-1 activation. Vascular calcification is an actively regulated process similar to bone formation involving cells and factors known to be important in the regulation of bone metabolism (reviewed in Dermer et al Trends Cardiovasc. Med. 4; 45-49, 1994). Regulators of osteoblastogenesis and/or osteoclastogenesis may modulate the degree of vessel wall calcification. The NAB proteins NAB1 and NAB2 (NGFI-A binding corepressors) interact with the conserved R1 domain of the Egr-1 and Egr-2 *trans*-activators (Svaren et al EMBO J., 17; 6010-6019 ,1998). It has previously been shown that NAB2 will repress the NGF-induced differentiation of PC12 cells (Qu,Z. et al J. Cell Biol. 142; 1075-1082, 1998) and Egr-1 mediated-activation of basic FGF (Svaren et al EMBO J., 17; 6010-6019, 1998).

One problem encountered in wound healing is the formation of hypertropic and keloid scars. This is extremely undesirable, particularly after cosmetic surgery. Tissue fibrosis (e.g. as applied to kidney, liver or skin) is manifested by accumulation of extracellular matrix. Dysregulated production of extracellular matrix which underlies the development of tissue fibrosis is regulated at least in part by a number of growth factors principally, but not restricted to TGFβ (Muir Eur. J. Plast. Surg. 21; 1-7, 1998, PDGF isoforms (Katou et al J. Pathol. 186; 201-208, 1998, Heldin et al in The molecular and cellular biology of wound repair, Clark, ed.; 249-264, 1996 Plenum Press) and VEGF (Jones et al Frontiers in Bioscience 4; D303-309, 1999). A therapy that would reduce, but not eliminate the expression of growth factors at the wound site would have significant impact on the healing of scar reduced tissue and improve quality of life.

International Patent Application number PCT.GB99.01722 describes the use of Egr-1 transcription factor in promoting wound healing. The present inventors have now found that administration of a polynucleotide encoding the transcriptional repressor NAB1 or NAB2: (a) represses Egr-1 mediated

activation of growth factors *in vitro*; (b) represses basal levels of expression of growth factor genes *in vitro;* and (c) at a site of wounding in a rodent, and subsequent expression thereof, reduces expression of TGF-β1 but not TGF-β3 and has application for reducing scarring during healing (e.g. Shah et al J. Cell Science 107; 1137-1157, 1994).

Therefore, the down-regulation of Egr-1 slows down the healing process and reduces the incidence of hypertrophic and keloid scar formation and psoriasis. Down-regulation of Egr-1 may also be of utility in the treatment of other disorders associated with wound healing, such as restenosis following percutaneous trans-luminal coronary angioplasty, modulation of vessel wall calcification and inhibition of cell proliferation in cancer.

Thus, according to one aspect of the invention, there is provided the use of a nucleic acid molecule comprising a sequence encoding an NAB1 or NAB2 polypeptide, or a biologically active fragment thereof, in the manufacture of a medicament for treatment of cell proliferative disorders associated with wound healing in a mammal, including human.

According to a further aspect of the invention, there is provided a method of treatment of cell proliferative disorders associated with wound healing in a mammal, including human, which comprises the administration to the mammal of a nucleic acid molecule comprising a sequence encoding an NAB1 or NAB2 polypeptide, or a biologically active fragment thereof.

According to a further aspect, the invention provides a nucleic acid molecule comprising a sequence encoding an NAB1 or NAB2 polypeptide or a biologically active fragment thereof for use in the treatment of cell proliferative disorders associated with wound healing.

As a further aspect, the invention extends to use of a combination of a nucleic acid molecule comprising a sequence encoding an NAB1 polypeptide and another comprising a sequence encoding an NAB2 polypeptide.

As a preferred aspect of the present invention, the cell proliferative disorders associated with wound healing are selected from hypertrophic and keloid scar formation, psoriasis, restenosis following percutaneous trans-luminal coronary angioplasty, vessel wall calcification and cell proliferation in cancer. As a particularly preferred aspect of the present invention, the cell proliferative disorders are hypertrophic and keloid scar formation.

The present invention, thus, relates to the therapeutic use in wound healing processes of polynucleotides encoding NAB1 or NAB2. The invention also relates to therapeutic use in wound healing processes of NAB1 or NAB2 itself, as described in greater detail below.

The invention relates to the use of NAB1 or NAB2 polypeptides and nucleic acid sequences encoding NAB1 or NAB2 polypeptides from any origin or species. The human DNA sequence is listed on Genbank under accession number U47007, which encodes for a 486 amino acid nuclear protein. Rat NAB1 is a 570 amino acid nuclear protein and is described in PNAS USA 92, 1995 p6873-6877. The rat DNA sequence is listed on Genbank under accession number U17253.

Mouse and human NAB2 protein sequences are described in molecular and Cellular Biology, 1996 16, 3545-3553. The human DNA sequence is listed on Genbank under accession number U48361.

References to NAB1 or NAB polypeptides and polynucleotides described hereinafter are generally applicable to the sequences of any origin, particularly the human sequences described above. As will be described below, the term NAB1 or NAB2 includes also variants, fragments and analogues of NAB1 or NAB2.

The following illustrative explanations are provided to facilitate understanding of certain terms used herein. The explanations are provided as a convenience and are not limitative of the invention.

Biologically active fragments of NAB1 or NAB2 as referred to herein are those fragments which have Egr-1 transcription repressing activity.

GENETIC ELEMENT generally means a polynucleotide comprising a region that encodes a polypeptide or a polynucleotide region that regulates replication, transcription or translation or other processes important to expression of the polypeptide in a host cell, or a polynucleotide comprising both a region that encodes a polypeptide and a region operably linked thereto that regulates expression. Genetic elements may be comprised within a vector that replicates as an episomal element; that is, as a molecule physically independent of the host cell genome. They may be comprised within plasmids. Genetic elements also may be comprised within a host cell genome; not in their natural state but, rather, following manipulation such as isolation, cloning and introduction into a host cell in the form of purified DNA or in a vector, among others.

HOST CELL is a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous polynucleotide sequence.

IDENTITY, as known in the art, is the relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. Identity can be readily calculated (*Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or two polypeptide sequences, the term is well known to skilled artisans (*Sequence Analysis in Molecular Biology*, von

Heinje, G., Academic Press, 1987; *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM *J. Applied Math., 48:* 1073 (1988). Methods commonly employed to determine identity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., SIAM *J. Applied Math., 48:*1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity are codified in computer programs. Preferred computer program methods to determine identity between two sequences include, but are not limited to, GCG program package (Devereux, J., et al., *Nucleic Acids Research 12(1):* 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S.F. et al., *J. Molec. Biol. 215:* 403 (1990)).

ISOLATED means altered "by the hand of man" from its natural state; *i.e.*, that, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide or a polypeptide naturally present in a living organism in its natural state is not "isolated", but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. As part of or following isolation, such polynucleotides can be joined to other polynucleotides, such as DNAs, for mutagenesis, to form fusion proteins, and for propagation or expression in a host, for instance. The isolated polynucleotides, alone or joined to other polynucleotide sequences such as in the form of vectors, can be introduced into host cells, in culture or in whole organisms. Introduced into host cells in culture or in whole organisms, such DNAs still would be isolated, as the term is used herein, because they would not be in their naturally occurring form or environment which are not naturally occurring compositions, and, therein remain isolated polynucleotides or polypeptides within the meaning of that term as it is employed herein.

POLYNUCLEOTIDE(S) generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA or cDNA. Thus, for instance, polynucleotides as used herein refers to, among others, single-and double-stranded DNA, DNA that is a

mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded, or a mixture of single- and double-stranded regions. In addition, polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term polynucleotide includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, *inter alia*. Polynucleotides embraces short polynucleotides often referred to as oligonucleotide(s).

POLYPEPTIDES, as used herein, includes all polypeptides as described below. The basic structure of polypeptides is well known and has been described in innumerable textbooks and other publications in the art. In this context, the term is used herein to refer to any peptide or protein comprising two or more amino acids joined to each other in a linear chain by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. It will be appreciated that polypeptides often contain amino acids other than the 20 amino acids

commonly referred to as the 20 naturally occurring amino acids, and that many amino acids, including the terminal amino acids, may be modified in a given polypeptide, either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques which are well known to the art. Even the common modifications that occur naturally in polypeptides are too numerous to list exhaustively here, but they are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art.

Among the known modifications which may be present in polypeptides for use in the present invention are, to name an illustrative few, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. Such modifications are well known to those of skill and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as, for instance *PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as, for example, those provided by Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in *POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS*, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter et al., *Meth. Enzymol. 182*:626-646 (1990) and Rattan et al., *Protein Synthesis: Posttranslational Modifications and Aging*, Ann. N.Y. Acad. Sci. 663: 48-62

(1992). It will be appreciated, as is well known and as noted above, that polypeptides are not always entirely linear. For instance, polypeptides may be generally as a result of posttranslational events, including natural processing event and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translation natural process and by entirely synthetic methods, as well. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. In fact, blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, is common in naturally occurring and synthetic polypeptides and such modifications may be present in polypeptides of the present invention, as well. For instance, the amino terminal residue of polypeptides made in *E. coli* or other cells, prior to proteolytic processing, almost invariably will be N-formylmethionine. During post-translational modification of the peptide, a methionine residue at the NH_2-terminus may be deleted. Accordingly, this invention contemplates the use of both the methionine-containing and the methioninelessamino terminal variants of the protein of the invention. The modifications that occur in a polypeptide often will be a function of how it is made. For polypeptides made by expressing a cloned gene in a host, for instance, the nature and extent of the modifications in large part will be determined by the host cell posttranslational modification capacity and the modification signals present in the polypeptide amino acid sequence. For instance, as is well known, glycosylation often does not occur in bacterial hosts such as, for example, *E. coli*. Accordingly, when glycosylation is desired, a polypeptide should be expressed in a glycosylating host, generally a eukaryotic cell. Insect cell often carry out the same posttranslational glycosylations as mammalian cells and, for this reason, insect cell expression systems have been developed to express efficiently mammalian proteins having native patterns of glycosylation, *inter alia*. Similar considerations apply to other modifications. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. In general, as used herein, the term polypeptide encompasses all such modifications, particularly

those that are present in polypeptides synthesized recombinantly by expressing a polynucleotide in a host cell.

VARIANT(S) of polynucleotides or polypeptides, as the term is used herein, are polynucleotides or polypeptides that differ from a reference polynucleotide or polypeptide, respectively. Variants in this sense are described below and elsewhere in the present disclosure in greater detail. (1) A polynucleotide that differs in nucleotide sequence from another, reference polynucleotide. Generally, differences are limited so that the nucleotide sequences of the reference and the variant are closely similar overall and, in many regions, identical. As noted below, changes in the nucleotide sequence of the variant may be silent. That is, they may not alter the amino acids encoded by the polynucleotide. Where alterations are limited to silent changes of this type a variant will encode a polypeptide with the same amino acid sequence as the reference. Also as noted below, changes in the nucleotide sequence of the variant may alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Such nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. (2) A polypeptide that differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference and the variant are closely similar overall and, in many region, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

The invention relates to therapeutic uses of a nucleic acid molecule comprising a sequence which encodes an NAB1 or NAB2 polypeptide, or a combination thereof. The invention relates also to therapeutic uses of fragments of said polynucleotide sequence which encode biologically active fragments of an NAB1 or NAB2 polypeptide, or variants of the polynucleotide sequence which, by virtue of the degeneracy of the genetic code, encode functional, ie biologically active fragments of NAB1 or NAB2, and to functionally equivalent allelic variants and related sequences modified by

single or multiple base substitution, addition and/or deletion which encode polypeptides having NAB1 or NAB2 activity.

These may be obtained by standard cloning procedures known to the persons skilled in the art.

Polynucleotides encoding NAB1 or NAB2 transcriptional repressor proteins may be in the form of DNA, cDNA or RNA such as mRNA obtained by cloning or produced by chemical synthetic techniques. The DNA may be single or double stranded. Single stranded DNA may be the coding or sense strand or it may be the non-coding or anti-sense strand. For therapeutic use, the polynucleotide is in a form capable of being expressed to a functional NAB1 or NAB2 transcription repressor protein at the wound site in the subject to be treated. The polynucleotides may also be used for *in vitro* production of an NAB1 or NAB2 polypeptide for administration in a further therapeutic aspect of the invention as described in detail below.

Polynucleotides of the present invention which encode a polypeptide of NAB1 or NAB2 may include, but are not limited to the coding sequence for NAB1 or NAB2 polypeptide, or biologically active fragments thereof. Thus, the polynucleotide may be provided together with additional, non-coding sequences, including for example, but not limited to non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription (including termination signals, for example), ribosome binding, mRNA stability elements, and additional coding sequence which encode additional amino acids, such as those which provide additional functionalities. Polynucleotides of the invention also include, but are not limited to, polynucleotides comprising a structural gene for NAB1 or NAB2 and its naturally associated genetic elements.

In accordance with the foregoing, the term "polynucleotide encoding a polypeptide" as used herein encompasses polynucleotides which include a sequence encoding a polypeptide of NAB1 or NAB2. The term encompasses polynucleotides that include a single continuous region or discontinuous regions encoding the polypeptide (for example, interrupted by integrated

phage or insertion sequence or editing) together with additional regions, that also may contain coding and/or non-coding sequences.

The present invention further relates to variants of the herein above described polynucleotides which encode fragments, analogs and derivatives of the polypeptide. A variant of the polynucleotide may be a naturally occurring variant such as a naturally occurring allelic variant, or it may be a variant that is not known to occur naturally. Such non-naturally occurring variants of the polynucleotide may be made by mutagenesis techniques, including those applied to polynucleotides, cells or organisms.

Among variants in this regard are variants that differ from the aforementioned polynucleotides by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions.

Further preferred embodiments of the invention are polynucleotides that are at least 70% identical over their entire length to a polynucleotide encoding polypeptides having the amino acid sequence described hereinbefore and polynucleotides which are complementary to such polynucleotides. Alternatively, most highly preferred are polynucleotides that comprise a region that is at least 80% identical over their entire length to a polynucleotide encoding a polypeptide of the present invention. In this regard, polynucleotides at least 90% identical over their entire length to the same are particularly preferred, and among these particularly preferred polynucleotides, those with at least 95% are especially preferred. Furthermore, those with at least 97% are highly preferred among those with at least 95%, and among these those with at least 98% and at least 99% are particularly highly preferred, with at least 99% being the more preferred.

Preferred embodiments in this respect, moreover, are polynucleotides which encode polypeptides which retain substantially the same biological function or activity as the mature NAB1 or NAB2 polypeptide encoded by the DNA

sequences described hereinbefore (Genbank accession numbers U47007 and U48361).

The present invention further relates to polynucleotides that hybridize to the herein above-described sequences. In this regard, the present invention especially relates to polynucleotides which hybridize under stringent conditions to the herein above-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur if there is at least 95% and preferably at least 97% identity between the sequences. Preferably the sequences which hybridise in this manner to the sequence of the invention encode a polypeptide having the biological activity of NAB1 or NAB2.

The polynucleotides may encode a polypeptide which is the mature protein plus additional amino or carboxyl-terminal amino acids. Such additional sequences may play a role for example, may lengthen or shorten protein half-life or may facilitate manipulation of a protein for assay or production, among other things. As generally is the case *in vivo*, the additional amino acids may be processed away from the mature protein by cellular enzymes.

Polynucleotides for use in the gene therapy aspect of the invention may be provided alone, or as part of a vector, such as an expression vector, examples of which are well known in the art.

An NAB1 or NAB2 encoding polynucleotide may be used therapeutically in the method of the invention by way of gene therapy in which the polynucleotide is administered to a wound site or to other tissues in need of healing in a form in which it is capable of directing the production of NAB1 or NAB2, or a biologically active fragment thereof, in situ.

Preferably in gene therapy, the polynucleotide is administered such that it is expressed in the subject to be treated for example in the form of a recombinant DNA molecule comprising a polynucleotide encoding NAB1 or NAB2 operatively linked to a nucleic acid sequence which controls expression such as in an expression vector. Such a vector will thus include appropriate

transcriptional control signals including a promoter region capable of expressing the coding sequence, said promoter being operable in the subject to be treated. Thus for human gene therapy, the promoter, which term includes not only the sequence necessary to direct RNA polymerase to the transcriptional start site, but also, if appropriate, other operating or controlling sequences including enhancers, is preferably a human promoter sequence from a human gene, or from a gene which is typically expressed in humans, such as the promoter from human CMV. Among known eukaryotic promoters suitable in this regard are the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus ("RSV"), and metallothionein promoters, such as the mouse metallothionein-I promoter.

A polynucleotide sequence and transcriptional control sequence may be provided cloned into a replicable plasmid vector, based on commercially available plasmids, such as pBR322, or may be constructed from available plasmids by routine application of well known, published procedures.

The vector may also include transcriptional control signals, situated 3' to the NAB1 or NAB2 encoding sequence, and also polyadenylation signals, recognisable in the subject to be treated, such as , for example, the corresponding sequences from viruses such as, for human treatment, the SV40 virus. Other transcriptional controlling sequences are well known in the art and may be used.

The expression vectors may also include selectable markers, such as for antibiotic resistance, which enable the vectors to be propagated.

Expression vectors capable *in situ* of synthesising NAB1 or NAB2 may be introduced into the wound site directly by physical methods. Examples of these include topical application of the 'naked' nucleic acid vector in an appropriate vehicle, for example, in solution in a pharmaceutically acceptable excipient such as phosphate buffered saline (PBS), or administration of the vector by physical methods such as particle bombardment, also known as

'gene gun' technology, according to methods known in the art, e.g. as described in US patent number 5371015 in which inert particles, such as gold beads coated with the vector are accelerated at speeds sufficient to enable them to penetrate the surface at the wound site, e.g. skin cells, by means of discharge under high pressure from a projecting device.

Other physical methods of administering the DNA directly to the recipient include ultrasound, electrical stimulation and electroporation.

An NAB1 or NAB2 encoding nucleic acid sequence for use in the therapy of the invention may also be administered by means of delivery vectors. These include viral delivery vectors, such as adenovirus or retrovirus delivery vectors known in the art.

Other non-viral delivery vectors include lipid delivery vectors, including liposome delivery vehicles, known in the art.

An NAB1 or NAB2 encoding nucleic acid sequence may also be administered to the wound site by means of transformed host cells. Such cells include cells harvested from the subject, into which the nucleic acid sequence is introduced by gene transfer methods known in the art, followed by growth of the transformed cells in culture and grafting to the subject.

Expression constructs such as those described above may be used in a variety of ways in the therapy of the present invention. Thus they may be directly administered to the wound site in the subject, or they may be used to prepare a recombinant NAB1 or NAB2 polypeptide itself which can then be administered to the wound site as is discussed in more detail below. The invention also relates to host cells which are genetically engineered with constructs which comprise NAB1 or NAB2 polynucleotide or polynucleotides of the present invention or genetic elements defined hereinabove, and to the uses of these vectors and cells in the therapeutic methods of the invention. These constructs may be used per se in the therapeutic methods of the invention or they may be used to prepare an NAB1 or NAB2 polypeptide for

use in the therapeutic methods of the invention described in greater detail below.

The vector may be, for example, a plasmid vector, a single or double-stranded phage vector, a single or double-stranded RNA or DNA viral vector, depending upon whether the vector is to be administered directly at the wound site (ie for in situ synthesis of NAB1 or NAB2), or is to be used for synthesis of recombinant NAB1 or NAB2. Starting plasmids disclosed herein are either commercially available, publicly available, or can be constructed from available plasmids by routine application of well known, published procedures. Many plasmids and other cloning and expression vectors that can be used in accordance with the present invention are well known and readily available to those of skill in the art.

Generally, vectors for expressing an NAB1 or NAB2 polypeptide for use in the invention comprise *cis*-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate *trans*-acting factors, either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain embodiments in this regard, the vectors provide for specific expression. For production of recombinant NAB1 or NAB2, such specific expression may be inducible expression or expression only in certain types of cells or both inducible and cell-specific. Particularly preferred among inducible vectors are vectors that can be induced for expression by environmental factors that are easy to manipulate, such as temperature and nutrient additives. A variety of vectors suitable to this aspect of the invention, including constitutive and inducible expression vectors for use in prokaryotic and eukaryotic hosts, are well known and employed routinely by those of skill in the art.

A great variety of expression vectors can be used to express NAB1 or NAB2 of the invention. Such vectors include, among others, chromosomal, episomal and virus-derived vectors, *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from

insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

The appropriate DNA sequence may be inserted into the vector by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

The nucleic acid sequence in the expression vector is operatively linked to appropriate expression control sequence(s), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include, but are not limited to, the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, for recombinant expression, and the SV40 early and late promoters and promoters of retroviral LTRs for *in situ* expression.

In general, expression constructs will contain sites for transcription initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, in accordance with many commonly practiced procedures, such regions will operate by controlling transcription, such as transcription factors, repressor binding sites and termination, among others.

Vectors for propagation and expression generally will include selectable markers and amplification regions, such as, for example, those set forth in Sambrook *et al.*, *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

Representative examples of appropriate hosts for recombinant expression of NAB1 or NAB2 include bacterial cells, such as streptococci, staphylococci, *E. coli*, streptomyces and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293 and Bowes melanoma cells; and plant cells.

The following vectors, which are commercially available, are provided by way of example. Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia, and pBR322 (ATCC 37017). Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors which can be used both for recombinant expression and for in situ expression are listed solely by way of illustration of the many commercially available and well known vectors that are available to those of skill in the art for use in accordance with this aspect of the present invention. It will be appreciated that any other plasmid or vector suitable for, for example, introduction, maintenance, propagation or expression of a polynucleotide or polypeptide of the invention in a host may be used in this aspect of the invention.

Examples of vectors for use in this aspect of the invention include expression vectors in which an NAB1 or NAB2 cDNA sequence is inserted in a plasmid whereby gene expression is driven from the human immediate early cytomegalovirus enhancer-promoter (Foecking and Hofstetter, Cell, 45, 101-

105, 1986). Such expression plasmids may contain SV40 RNA processing signals such as polyadenylation and termination signals. Expression constructs which use the CMV promoter and that are commercially available are pCDM8, pcDNA1 and derivatives, pcDNA3 and derivatives (Invitrogen). Other expression vectors available which may be used are pSVK3 and pSVL which contain the SV40 promoter and mRNA splice site and polyadenylation signals from SV40 (pSVK3) and SV40 VP1 processing signals (pSVL; vectors from Pharmacia).

Promoter regions can be selected from any desired gene using vectors that contain a reporter transcription unit lacking a promoter region, such as a chloramphenicol acetyl transferase ("CAT") transcription unit, downstream of restriction site or sites for introducing a candidate promoter fragment; *i.e.*, a fragment that may contain a promoter. As is well known, introduction into the vector of a promoter-containing fragment at the restriction site upstream of the *cat* gene engenders production of CAT activity, which can be detected by standard CAT assays. Vectors suitable to this end are well known and readily available, such as pKK232-8 and pCM7. Promoters for expression of polynucleotides of the present invention include not only well known and readily available promoters, but also promoters that readily may be obtained by the foregoing technique, using a reporter gene; for in situ expression, such a promoter should desirably be recognised in the subject to be treated.

Among known prokaryotic promoters suitable for expression of polynucleotides and polypeptides in accordance with the present invention are the *E. coli* lacI and lacZ and promoters, the T3 and T7 promoters, the *gpt* promoter, the lambda PR, PL promoters and the trp promoter.

Recombinant expression vectors will include, for example, origins of replication, a promoter preferably derived from a highly-expressed gene to direct transcription of a downstream structural sequence, and a selectable marker to permit isolation of vector containing cells after exposure to the vector.

Polynucleotides of the invention, encoding the heterologous structural sequence of a polypeptide of the invention generally will be inserted into the vector using standard techniques so that it is operably linked to the promoter for expression. The polynucleotide will be positioned so that the transcription start site is located appropriately 5' to a ribosome binding site. The ribosome binding site will be 5' to the AUG that initiates translation of the polypeptide to be expressed.

Generally, there will be no other open reading frames that begin with an initiation codon, usually AUG, and lie between the ribosome binding site and the initiation codon. Also, generally, there will be a translation stop codon at the end of the polypeptide and there will be a polyadenylation signal in constructs for use in eukaryotic hosts. Transcription termination signal appropriately disposed at the 3' end of the transcribed region may also be included in the polynucleotide construct.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide when recombinantly synthesised. These signals may be endogenous to the polypeptide or they may be heterologous signals.

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N- or C-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, region also may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability or to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunolglobulin that is useful to solubilize or purify polypeptides. Cells typically then are harvested by centrifugation,

disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art.

Mammalian expression vectors may comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation regions, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences that are necessary for expression.

For preparing NAB1 or NAB2 polypeptides for use in the invention genetically engineered host cells may be used. Introduction of a polynucleotides into the host cell can be affected by calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., *BASIC METHODS IN MOLECULAR BIOLOGY*, (1986) and Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Mature proteins can be expressed in host cells including mammalian cells such as CHO cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

The polypeptide can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

For therapy, an NAB1 or NAB2 encoding polynucleotide, eg in the form of a recombinant vector, may be purified by techniques known in the art, such as by means of column chromatography as described in Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

As indicated above, an NAB1 or NAB2 polypeptide may be administered at the site of wounding either as an NAB1 or NAB2 encoding nucleic acid which is transcribed and translated to NAB1 or NAB 2 at the wound site itself in a form of gene therapy, or the transcription repressor protein itself may be directly administered.

Thus according to a further aspect of the invention there is provided the use of an NAB1 or NAB2 polypeptide, or a biologically active fragment thereof, in the manufacture of a medicament for the treatment of cell proliferative disorders in a mammal, including human.

According to a further aspect of the invention there is provided a method of treatment of cell proliferative disorders in a mammal, including human, which comprises the administration to the mammal of a therapeutically effective amount of an NAB1 or NAB2 polypeptide, or a biologically active fragment thereof.

Thus viewed from a further aspect, the invention provides the use of an NAB1 or NAB2 polypeptide, or a biologically active fragment thereof, for use in the treatment of wounds and in wound healing.

As used herein, the term "NAB1 or NAB2 polypeptide" includes naturally and recombinantly produced NAB1 or NAB2, natural, synthetic and biologically active polypeptide analogues or variants or derivatives thereof or biologically active fragments thereof and variants, derivatives and analogues of said fragments.

NAB1 or NAB2 protein products, including biologically active fragments of NAB1 or NAB2, may be generated and/or isolated by general techniques known in the art.

NAB1 or NAB2 and the aforementioned fragments and derivatives thereof for use in the therapy of the invention may be extracted from natural sources by methods known in the art. Such methods include purification by means of sequence specific DNA affinity chromatography using methods such as those described in Briggs et al, Science 234, 47-52, 1986, using a DNA binding oligonucleotide which recognises NAB1 or NAB2. The polypeptide may also be prepared by methods of recombinant DNA technology known to the art as described above, ie by expression in host cells of the constructs described. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

The invention also relates to uses of fragments, analogs and derivatives of NAB1 or NAB2. The terms "fragment", "derivative" and "analog" means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The fragment, derivative or analog of the polypeptide may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid

residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Among preferred variants are those that vary from naturally occurring NAB1 or NAB2 by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

Further particularly preferred in this regard are variants, analogs, derivatives and fragments, and variants, analogs and derivatives of the fragments, having the amino acid sequence of the polypeptide in which several, a few, 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues are substituted, deleted or added, in any combination. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the polypeptide of the present invention. Also especially preferred in this regard are conservative substitutions.

Particularly preferred fragments are biologically active fragments ie. fragments which retain the wound healing properties of the parent polypeptide.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

NAB1 or NAB2 polypeptides for use in the present invention include NAB1 or NAB2 polypeptide as well as polypeptides which have at least 70% identity preferably at least 80% identity to and more preferably at least 90% more identity and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide sequences described hereinabove and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The invention also relates to the use of fragments of an NAB1 or NAB2 polypeptide defined hereinabove and fragments of varients and derivatives thereof.

In this regard a fragment is a polypeptide having an amino acid sequence that entirely is the same as part but not all of the amino acid sequence of NAB1 or NAB2 polypeptides and variants or derivatives thereof.

Such fragments may be "free-standing", *i.e.*, not part of or fused to other amino acids or polypeptides, or they may be comprised within a larger polypeptide of which they form a part or region. When comprised within a larger polypeptide, the presently discussed fragments most preferably form a single continuous region. However, several fragments may be comprised within a single larger polypeptide. For instance, certain preferred embodiments relate to a fragment of a polypeptide of the present invention comprised within a precursor polypeptide designed for expression in a host

and having heterologous pre- and pro-polypeptide regions fused to the amino terminus of the fragment and an additional region fused to the carboxyl terminus of the fragment. Therefore, fragments in one aspect of the meaning intended herein, refers to the portion or portions of a fusion polypeptide or fusion protein derived froma polypeptide of the present invention.

Also preferred in this aspect of the invention are fragments characterized by structural or functional attributes of the polypeptide of the present invention. Preferred embodiments of the invention in this regard include fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions of the polypeptide of the present invention, and combinations of such fragments.

Preferred regions are those that mediate activities of the polypeptide of the present invention. Most highly preferred in this regard are fragments that have a chemical, biological or other activity of the polypeptide of the present invention, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Further preferred polypeptide fragments are those that comprise or contain antigenic or immunogenic determinants in an animal, especially in a human.

It will be appreciated that the invention also relates to, among others, polynucleotides encoding the aforementioned fragments, polynucleotides that hybridize to polynucleotides encoding the fragments, particularly those that hybridize under stringent conditions, and polynucleotides, such as PCR primers, for amplifying polynucleotides that encode the fragments. In these regards, preferred polynucleotides are those that correspond to the preferred fragments, as discussed above.

In further embodiments of this aspect of the invention include biologically, prophylactically, clinically or therapeutically useful variants, analogues or derivatives thereof, or fragments thereof, including fragments of the variants,

analogues and derivatives and compositions comprising the same. Biologically active varients, analogues or fragments are included in the scope of the present invention.

The invention also relates to compositions comprising the polynucleotides or polypeptides discussed above. Therefore, polynucleotides or polypeptides of the present invention may be employed in combination with a pharmaceutically acceptable carrier or carriers.

Such carriers may include, but are not limited to saline, buffered saline, dextrose, water, glycerol, ethanol and combinations thereof.

Polypeptides and polynucleotides may be employed in the present invention may alone or in conjunction with other compounds, such as therapeutic compounds.

The pharmaceutical compositions may be administered in any effective, convenient manner effective for targeting wound sites including, for instance, administration by topical, intravenous, intramuscular, intranasal or intradermal routes among others.

In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

Alternatively the composition may be formulated for topical application for example in the form of ointments, creams, lotions, eye ointments, eye drops, ear drops, mouthwash, impregnated dressings and sutures and aerosols, and may contain appropriate conventional additives, including, for example, preservatives, solvents to assist drug penetration, and emollients in ointments and creams. Such topical formulations may also contain compatible conventional carriers, for example cream or ointment bases, and ethanol or oleyl alcohol for lotions. Such carriers may constitute from about 1% to about 98% by weight of the formulation; more usually they will constitute up to about 80% by weight of the formulation.

For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be from 0.01 mg/kg to 10 mg/kg, typically around 1 mg/kg. The physician in any event will determine the actual dosage which will be most suitable for an individual and will vary with the age, weight and response of the particular individual. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention. Ultimately, the dose selected by the skilled person will have the function of reducing cell proliferation without preventing wound healing.

As a yet further aspect, there is provided a pharmaceutical composition comprising an NAB1 or NAB2 polypeptide or a nucleic acid molecule comprising a sequence encoding a NAB1 or NAB2 polypeptide, together with one or more pharmaceutically acceptable carriers thereof.

The therapeutic advantage of using transcription repressor proteins in wound healing is in the deactivation of multiple target genes which promote accelerated healing. NAB1 or NAB2 is naturally activated in response to wounding and augmenting the natural response is also an advantage. The treatment is DNA based and it provides a reliable and reproducible delivery system.

Where an NAB1 or NAB2 polynucleotide is used in the therapeutic method of the invention, the polynucleotide may be used as part of an expression construct eg. in the form of an expression vector. In such a method, the construct is introduced at the wound site where NAB1 or NAB2 is produced in situ. The constructs used may be standard vectors and/or gene delivery systems, such as liposomes, receptor-mediated delivery systems and viral vectors.

The present invention is suitable for all aspects of wound healing including limb ulcerations in diabetes and peripheral arterial occlusive disease, post-operative scarring, burns, psoriasis, inhibition of restenosis following

percutaneous trans-luminal coronary angioplasty, modulation of vessel wall calcification and inhibition of cell proliferation in cancer.

As described above, NAB1 or NAB2 polypeptides or nucleic acids of the present invention may be administered locally to the site of tissue damage by any convenient method e.g. by topical administration. A preferred method of delivery of nucleic acid products is using gene gun technology in which the Egr-1 isolated nucleic acid molecule e.g. in the form of cDNA or in an expression vector is immobilised on gold particles and fired directly at the site of wounding. Thus, as a preferred aspect of the present invention, there is provided the use of a nucleic acid molecule comprising a sequence encoding an NAB1 or NAB2 polypeptide in a gene gun for the treatment of cell proliferative disorders associated with wound healing. Further, there is provided a composition suitable for gene gun therapy comprising an NAB1 or NAB2 encoding sequence immobilised on gold particles.

The present invention will now be described by way of the following non-limiting examples with reference to the accompanying figures, wherein:

Figures 1a) - 1d). show repression of Egr-1 mediated activation of PDGF-AB, TGFβ, HGF and VEGF respectively;
Figure 2 describes NAB2 trans-repression of Egr-1 mediated HGF production in HVSMC;
Figure 3 describes the effect of NAB2 on Egr-1 driven angiogenesis;
Figure 4a describes the effect of NAB2 transfection on linear incisional wound contraction at 7 days post-wounding;
Figure 4b describes the effect of NAB2 transfection on the levels of growth factors in the epidermis of 7 day rat incisional wounds;
Figure 4c describes the effect of NAB2 transfection on the levels of growth factors in the granulation tissue of 7 day rat incisional wounds; and
Figure 4d describes the effect of NAB2 transfection on angiogenesis in 7 day rat incisional wounds.

Examples

Example 1

Use of NAB2 to repress Egr-1 mediated trans-activation of growth factors

1.1 Methods

Human vascular smooth muscle cells (HVSMC; Clonetics) were cultured according to the manufacturer's recommendations. Cells were cultured for transfection in 6-well microtitre plates (Costar). An expression plasmid comprising the NAB2 cDNA driven from the human cytomegalovirus promoter (hCMV; Svaren et al Mol. Cell. Biol., 16; 3545-3553, 1996) was transfected together with an expression plasmid comprising the Egr-1 cDNA (Houston et al Arterioscler. Thromb. Vasc. Biol., 19; 281-289, 1999) into HVSMC at the ratios described in the figures using FUGENE (Boehringer Mannheim). A 3:1 ratio (v/w) of FUGENE:DNA was used for all experiments and transfection was normalised using a CMV driven β-galactosidase expression plasmid. Secreted growth factors were detected in tissue culture medium by ELISA (R&D Systems) using appropriate controls.

1.2 Results

Figures 1a). to 1d). show repression of Egr-1 mediated activation of PDGF-AB, TGFβ, HGF and VEGF respectively. 6 µg of Egr-1 expression plasmid was contransfected either alone (Figure 1a, Figure 1b and Figure 1d) or with 500, 100 or 25 ng NAB2 (Figure 1a and Figure 1d). For PDGF-AB, TGFβ and VEGF there was a small increase in the quantity of detected secreted growth factor when Egr-1 was transfected alone. On co-transfection with NAB2, there was complete ablation of the response of PDGF-AB to Egr-1 activation and reduction of basal expression leading to a five fold decrease in the total production of PDGF-AB (Figure 1a). NAB2 transfection completely ablated the response of TGFβ to Egr-1 activation and caused a 30% reduction in the production of total TGFβ (Figure 1b). Using the DNA concentrations shown in the Figure, Egr-1 transfection caused a 50% increase the production of HGF which was partially blocked by co-transfection of a low dose of NAB2 and completely blocked by a higher dose (Figure 1c). The greatest effects were seen at day 1 after transfection although inhibitory effects of NAB2 were apparent at days 2 and 3. NAB2 transfection, as with PDGF-AB and TGFβ

blocked Egr-1 mediated activation of VEGF and caused a 40% decrease in the amount of total VEGF produced (Figure 1d).

1.3 Conclusion

These data show that the Egr-1 supressor NAB2 can block growth factor activation mediated by Egr-1, such as is found at sites of acute injury.

Example 2

Use of NAB2 to repress basal levels of growth factors

2.1 Methods

Cell culture, transfection and detection of growth factors was carried out as described in section 1.1 above. NAB2 expression vector was transfected at final concentrations of 0, 250, 500, 1000 or 3000ng.

2.2 Results

As shown in Figure 2a, transfection of NAB2 into HVSMC caused a drastic reduction in the production of PDGF-AB. At the highest dose of NAB2, a 10-fold reduction in PDGF-AB was obtained.

2.3 Conclusion

These data show that the Egr-1 supressor can reduce the basal level of growth factors produced from both PDGF-AB promoters.

Example 3

Use of NAB to repress induction of angiogenesis *in vitro*

3.1 Methods

NAB2 expression constructs (wild type and dominant negative) driven from the hCMV promoter have been described (Svaren et al, EMBO J. 17; 6010-6019, 1998). We have shown that transfection of the Egr-1 transcription factor when expressed from the hCMV promoter promoted angiogenesis (Patent filing PG3412). In these experiments DNA was transfected into an angiogenesis kit as supplied and maintained according to the manufacturer's instructions (TCS Biologicals). VEGF protein (2ng/ml) was used as a positive

control and suramin (20 μM) as an angiogenesis inhibitor. CMV Egr-1 DNA was transfected at 0.5 μg per well in triplicate in a 24 well microtitre plate using Mirus Transit reagent (Cambridge Biosciences) at a ratio of 2:1 v/w DNA. To assay the potential of NAB2 to repress Egr-1 mediated angiogenesis, 0.5 mg Egr-1 DNA was co-transfected with 10, 25 or 100 ng per well NAB2 expression plasmid in the absence or presence of 100ng of a NAB2 dominant negative expression plasmid. After 11 days co-culture angiogenesis was determined by staining of cells for the endothelial cell marker PECAM-1 and visualisation using BCIP/NBT substrate.

Representative images of tubule formation using all four doses of Egr-1 expression plasmid together with VEGF (positive control) and suramin (negative control) were captured and processed by image analysis using Quantimet 600 image analyser and associated software.

3.2 Results

Egr-1 DNA has been shown to be pro-angiogenic in International Patent Application Number PCT.GB99.01722 (as shown in column 4 of Figure 3a.). As shown in Figure 3a, co-transfection with NAB2 gave a dose dependent reduction in the ability of Egr-1 to induce angiogenesis, which was partially ablated by co-transfection with the NAB2 dominant negative.

3.3 Conclusion

NAB2 may be used to block angiogenesis in the background of acute injury, an example of which is growth factor induction by Egr-1.

Example 4

Use of NAB2 to reduce scarring in a rodent incisional model of wounding

4.1 Methods

4.1.1 The effect of NAB2 transfection using the gene gun on the growth factor levels of rat incisional wounds

NAB2 transfection into rodent incisional wounds has the potential of being anti-scarring through its direct suppressive action on the expression of key scarring growth factors, namely TGFβ1. In this experiment, NAB2 cDNA was transfected into rat incisional wounds using the Biorad gene gun and the effects on healing and growth factor levels assessed using routine histology and immuncytochemistry.

4.1.2 Particle Mediated Gene Transfer

Eight male Sprague Dawley rats weighing ~250g were anesthetised under isoflorane in a 2:1 mixture of oxygen/nitrous oxide. Two sites of transfection (5 cm from the base of the skull 1.5 cm either side of the midline) and 2 control sites (8cm from the base of the skull, 1.5cm either side of the midline) on the rat dorsum were prepared by firstly clipping the pelt, then shaving with a razor. One transfection was carried out at each transfection site by accelerating plasmid/gold complexes of either NAB2 or Egr-1 (positive control for growth factor activation) into the skin at 350psi. Typically 0.5-1.5 μg DNA was delivered per transfection. At one control site, gold particles (without DNA) were accelerated into the skin at 350psi; the other control site was left unmanipulated. The sites of transfection and control sites were rotated clockwise within each additional animal to control for anterior-posterior differences in the healing of rodent wounds.

4.1.3 Incisional Wound Healing Model

Twenty four hours post transfection, animals were anaesthetised and a 1cm linear full thickness incision made parallel to the spine, using a scalpel blade at the exact sites of transfection. Animals were allowed to recover from anaesthesia and the wound left to heal free of sutures. At 7 days post wounding all 8 animals were killed and the wounds dissected out and harvested for routine histology and immunohistochemistry

4.1.4 Histological Analysis

Each wound per time point after dissection was bisected horizontally. One half was placed in 4% paraformaldehyde for 24 hours and processed for wax histology. 5mm sections from each wound were cut using a microtome and

the sections stained with van Geison. The sections were observed using light microscopy and the effect of NAB2 on the healing response assessed.

4.1.5 Immunhistochemistry

Once frozen in OCT, the second half of each wound was sectioned at 7mm using a cryostat. Two sections from each wound were fixed in ice-cold acetone and fluorescent immunostaining performed with primary antibodies to Egr-1, PDGF, TGFβ1, TGFβ3 and vWF using the following protocol.

1. Wash slides with PBS
2. Apply 30ml primary antibody for 1 hour
3. Wash 3x5 minutes in PBS
4. Apply 30ml secondary antibody (FITC directly linked) for 45 minutes
5. Wash 3x5 minutes in PBS
6. Slides mounted using aqueous mount

Immediately after immunostaining each slide was placed under a fluorescent microscope and the wound area captured using a x100 magnification. The image was intergrated and a threshold set to minimise backround. Area and intensity of staining was measured using image analysis and plotted graphically.

4.2 Results

4.2.1 Effect of NAB2 on rat incisional wound healing

4.2.1.1 Wound contraction and Histological analysis of 7 day wounds

According to Figure 4a, wounds treated with NAB2 contracted to a similar width as Egr-1 transfected and controls wounds and histologically showed similar granulation tissue maturity.

Conclusion

Delivery of NAB2 does not impair the rate of healing in a rodent incisional model of healing.

4.2.1.2 Growth Factor Profiles

Immunostaining for Egr-1, TGFβ1, TGFβ3 and PDGF-AB was performed on frozen cryosections of skin tissue at the wound site and the intensity of staining for each growth factor measured using image analysis. Two separate immunohistochemical measurements were made within the wound site. Sections were examined for growth factor expression in both the epidermis immediately above the wound and within the wound site (granulation tissue).

a) Positive staining for growth factors in the epidermis

As shown in Figure 4b, at 7 days post wounding NAB2 transfection markedly reduced the expression of TGFβ1 in the epidermis compared to Egr-1 and gold alone control. In comparison to the unmanipulated control, NAB2 transfection reduced the expression of TGFβ1 in the epidermis, whereas both Egr-1 and gold delivery activated the production of TGF β1. NAB2 transfection increased the levels of TGFβ3 in the epidermis compared to both gold alone and unmanipulated controls. NAB2 transfection did not markedly alter the epidermal expression of Egr-1 and PDGF.

b) Positive staining for growth factors in the granulation tissue

As shown in Figure 4c, at 7 days post wounding NAB2 transfection had no effect on the levels of Egr-1, PDGF-AB and TGFβ1 within the granulation tissue. However NAB2 increased the levels of TGFβ3 within the granulation tissue compared to both gold alone and unmanipulated controls.

Conclusion

NAB2 transfection of incisional wounds decreased the levels of TGFb1 in the epidermis and increased the levels of TGFβ3 in the epidermis and granulation tissue at 7 days post wounding. TGFβ1 is a known scarring agent while TGFβ3 has anti-scarring properties (Shah et al J. Cell Science 107; 1137-1157, 1994). Therefore delivery of NAB2 may have anti-scarring properties.

4.2.2 Effect of NAB2 on angiogenesis

At 7 days post-wounding, skin sections were stained and scored for vWF expression using immunohistochemistry and image analysis. Angiogenesis was quantified using von Willebrand factor immunostaining on wound

cryosections and image analysis to measure the area of positive staining within the wound site. As shown in Figure 4d, 7 days post wounding NAB2 transfected wounds had less new blood vessels compared to control (gold treated wounds). Egr-1 promoted angiogenesis in vivo, supporting the in vitro findings in Example 3.

Conclusion

NAB2 blocked the Egr-1 stimulated activation of angiogenesis in vivo, supporting a role of NAB2 as repressor of growth factor activation when driven by an acute stimulus, an example of which is Egr-1 activation of growth factor production.